ACCEPTED FOR PUBLICATION IN CANCER RESEARCH

TRANSCRIPTION OF GENES ENCODING GM-CSF, IL-3 AND IL-6 RECEPTORS AND LACK OF PROLIFERATIVE RESPONSE TO EXOGENOUS CYTOKINES IN NON-HEMATOPOIETIC HUMAN MALIGNANT CELL LINES[1]

Thierry Guillaume[2], Maryam Sekhavat, Daniel B. Rubinstein,

Oussama Hamdan, and Michel L. Symann

Laboratory of Experimental Oncology and Hematology

[T.G., M.S., O.H., M.L.S.]

Catholic University of Louvain, Brussels, Belgium,

Division of Hematology/Oncology, [DBR],

Boston University Medical Center, Boston, MA.

Running Title : Cytokine Receptor Gene Transcription in Malignant Cells.

Key Words : Cytokine receptors, cytokine stimulation, gene transcription, malignant cell lines, tumor growth.

1 This work was supported by the Oeuvre Belge du Cancer, the Association Sportive contre le Cancer and Fabriques de Tabac Réunies.

2 Correspondence : Dr. Thierry Guillaume, Laboratory of Experimental Oncology and Hematology, 54 Avenue Hippocrate, UCL 54.71, 1200 Brussels, Belgium.

3 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PCR, polymerase chain reaction; R, receptor; MEM, modified Eagle's medium; FCS, fetal calf serum.

2023251792

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

ABSTRACT

Studies in recent years have suggested that human tumor cell lines are capable of responding in vitro to hematopoietic growth factors. In the present study, we investigate the transcription of the α and β subunits of GM-CSF receptor (GM-CSFR), the α and β subunits of IL-3 receptor (IL-3R), the single subunit of IL-6 receptor (IL-6R) and its associated gp130 transduction protein by PCR amplification of reverse transcribed cellular mRNA in 34 malignant cell lines derived from a variety of histologic cell types. mRNA for only a single subunit polypeptide was found in a significant minority of cell lines (23%), while in 20% both the α and β subunits of either the GM-CSFR or the IL-3R were detected, distributed among a number of different histological cell types. Transcription of the gene encoding the IL-6R was found in 38% of cell lines, and all lines transcribed the gp130 transduction protein, consistent with previous observations on the ubiquity of that polypeptide. In order to test the in vitro effect of exogenously added growth factors on those malignant cell lines transcribing complete cytokine receptor, either GM-CSF, IL-3 or IL-6 was added in therapeutic concentrations (20-500 ng/ml) and cellular proliferation measured by incorporation of [^{3}H]-thymidine. No stimulation was seen at either 3 and 6 days of culture. Production of cytokine by these cell lines was investigated at the level of transcription and by assay of peptide product. None transcribed mRNA for either GM-CSF or IL-3, while 5 of six (STD, DOZ, ADE, Hep-2 and Detroit) expressed IL-6 mRNA. Of these latter, 2 (lines ADE and Hep-2) produced IL-6 as determined by bioassay, while none produced GM-CSF or IL-3 by ELISA. This suggests that in the case of GM-CSF and IL-3, failure to proliferate on addition of cytokine is not due to the prior presence of endogenous production. In contrast, at least a subset of malignant cell lines may involve a closed IL-6 autocrine loop saturating cell surface sites. These findings suggest that the ability to transcribe the genes encoding cytokine receptor is alone insufficient to render cells cytokine-responsive, and that malignant cells may lack the cellular machinery for cytokine-induced proliferation. This in turn suggests that therapeutic administration of either GM-CSF, IL-3 or IL-6 may involve no additional risk of tumor regrowth in vivo.

2023251793

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

INTRODUCTION

GM-CSF[3] and IL-3 are potent hematopoietic growth factors stimulating the proliferation and differentiation of both multipotent stem cells and lineage-committed hematopoietic cells (1,2). The transmembrane cell surface receptors which bind these growth factors and transduce their signal intracellularly have been cloned and characterized in recent years. The human GM-CSF receptor (GM-CSFR) is composed of two subunits, a low affinity alpha subunit specific for the GM-CSFR (3) and a beta subunit, which it shares in common with the IL-3 receptor (4). Together, these make up the 45 kDa receptor, and their coexpression results in high affinity GM-CSF binding (5). The IL-3 receptor (IL3R), likewise composed of two subunits, the common beta subunit and a recently cloned specific alpha subunit, has a combined molecular weight of 120 kDa (4,5). Interleukin-6 (IL-6) is a pleiotropic cytokine involved in immune and inflammatory processes in addition to its role in hematopoietic maturation (6). Its receptor (IL-6R), composed of a single unit with a molecular weight of 80 kDa, and an associated gp130 transduction protein have been cloned and characterized (7-9).

The influence of hematopoietic growth factors has recently been examined in a variety of human solid tumor cell lines, including those derived from small cell lung cancer, from melanoma, as well as from renal, colon gastric and ovarian carcinomas. Several reports suggest that GM-CSF, IL-3, and IL-6 might be capable of stimulating the growth of some of these malignant cell lines in vitro (10-21), although discrepancies have been found in comparing different studies of the same histologic tumor type and even among tumor cell lines derived from the very same source. These apparent inconsistencies likely arise from the biological heterogeneity of the tumors studied. What emerges, however, is the possibility that malignancies, or at least a subset of malignant cells, may owe part of their capacity to proliferate to stimulation by cytokines. Moreover, the stimulation may result from either a paracrine effect of cytokine produced by a source other than the tumor itself, or from a self-sustaining autocrine loop of tumor-produced cytokines (22-24).

Because of the increasing use of hematopoietic growth factors in clinical settings following chemotherapy and autologous bone marrow transplantation (25), there is concern that these

2023251794

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

stimulating factors, while promoting re-population of the bone marrow, might at the same time induce regrowth of residual tumor. In this report, we analyze the transcription of the genes encoding the GM-CSF, IL-3, and IL-6 receptors in 34 cell lines derived from a variety of human malignancies. RNA message for cytokine receptors was found to be transcribed to a greater degree than previously reported. However, despite evidence for transcription and expression of growth factor receptors by tumor cells, no indication was found that this results in cellular proliferation in vitro when the corresponding factors are exogenously added in a range of concentrations analogous to those administered therapeutically. More than just the presence of cell surface receptors may be required for a stimulatory response to cytokines; Failure to proliferate may be due to either an inability of tumor cells to bind cytokines in adequate concentrations or to an inability to properly transduce signal from the cell membrane to the cytosol. These in vitro findings imply that in vivo therapeutic administration of hematopoietic growth factors may entail no proliferation of tumor cells and no added risk of tumor recurrence clinically.

MATERIALS AND METHODS

Cell lines. Small cell lung cancer cell lines (OC1, OC2, OC3, FRA, STO, GIL, THO, BAT, DEV, VHE, FIC, DOZ) were established in our laboratory except for two cell lines (H69 and H128) kindly provided by the National Cancer Institute (Bethesda, MD), and were cultured in RPMI-HITES medium (26). Melanoma cell lines (DES, G43, WO, RENI, ADE) were developed in our laboratory and maintained in Dulbecco's medium with 20% Hepes, 4.5% glucose and 10% FCS. Head and neck carcinoma cell lines (Fadu, KB, Hep-2, Detroit 562, RPMI 2650) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in MEM with non-essential amino acids and 10% FCS. Ovarian neoplastic cell lines (AZ 224, AZ 382, AZ 364, AZ 303) obtained from Dr. J. De Grève (Division of Medical Genetics, Vrije Universiteit, Brussels) and prostatic carcinoma (DU145) cell line purchased from the ATCC were cultured in RPMI 1640 supplemented with 10% FCS. Two gastric cancer cell lines from the ATCC (AGS and KATO III) were cultured in Ham's F12 and RPMI 1640 media respectively. Finally, three kidney carcinoma cell lines obtained from the ATCC (CAKI I, CAKI II, A498) were maintained in

2023251795

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

McCoy's or MEM media. HL60 cells (a human promyelocytic cell line) (27), KG1 cells (28) and CESS cells (an EBV-transformed B cell line) (29) were used as positive controls.

PCR amplification and analysis of product. Total RNA was extracted from $5x10^6$ cells either using the phenol extraction method (30) or the guanidium method (31). cDNA was synthesized using 1 ug of total RNA with reverse transcriptase (BRL, Gaithersburg, MD), and random hexanucleotides (Pharmacia, Milwaukee, WI) in a 20 μl volume containing final concentrations of 50 mM Tris pH 8.3, 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT and 1 mM of each dNTP. 5 μl of the reverse transcriptase reaction was used for PCR amplification in a 50 μl volume containing 15 picomoles of primers, 1 nanomole of each dNTP, and 2.5 units of Taq DNA polymerase (BRL) in a final concentration of 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 2.5 mM $MgCl_2$. Reaction mixtures were subjected to 25 successive cycles consisting of heat denaturation (94°C, 1.5 min), annealing (55°C for 2 min for GM-CSFR alpha, GM-CSFR beta, IL-6R, IL-3, and IL-6, 60°C for IL-3R alpha, GM-CSF, and 65°C for human beta actin) and primer extension (72°C for 3 min). PCR products were size fractionated in 2% agarose gels, and blotted onto Genescreen Plus nylone membranes (Dupont NEN, Boston, MA). The blots were hybridized with gene-specific oligonucleotides probes internal to the paired PCR primers used in generating each product. Oligonucleotides were end-labelled by T4 polynucleotide kinase and gamma-[^{32}P] ATP (32). The sequences of each set of PCR primers and their corresponding gene-specific internal oligonucleotide probes are depicted in Table 1 (first citation of Table 1). Conditions of prehybridization, hybridization and washing were performed as previously described (32) at the temperatures indicated in Table 1.

First citation of Table 1

Assay for endogeneous cytokine production by malignant cells. Supernatants of malignant cell lines of at least 3 days growth were assayed for the presence of GM-CSF and IL-3 in 96 well-plates by ELISA as recommended by the manufacturer (Amersham International, UK). IL-6 activity was measured using the IL-6-dependent murine hybridoma 7TD1 as previously described (33).

2023251796

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

Cellular Proliferation Assay. Cells were cultured, in triplicate, in 100 μl of their respective medium containing FCS at a concentration lower than that used in the original cultures (3%) in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) at a concentration of 10^5 cells per ml. Human recombinant growth factors rGM-CSF, rIL-3 and rIL-6 (provided by Sandoz, Basel, Switzerland) were added at three different concentrations (20, 100, 500 ng/ml) to each set of triplicate wells at the initiation of the culture. After 3 and 6 days of culture, 0.5 uCi of [^{3}H]-thymidine (Dupont NEN) was added to each well and cells were harvested 24 hours later.

Statistical analysis. Values are expressed as the mean and ± standard error of the mean (SEM). The significance of differences between groups was calculated using the Student-Newman-Keuls test.

RESULTS

PCR amplification products of the cytokine receptor gene transcripts under study are depicted in Table 1. As indicated, the expected lengths of the gene transcripts were 530 bp for GM-CSFRα, 442 bp for IL-3Rα, 316 bp for the common β chain, 306 bp for IL-6R, 322 bp for GM-CSF, 344 bp for IL-3, and 459 bp for IL-6. In addition, the PCR primers corresponding to the IL-6 signal transducer yielded a 759 bp product and those for actin yielded a 519 bp product. Amplification with the actin primers confirmed the presence of intact cDNA in each sample. Hybridization of each PCR product to its corresponding gene-specific internal oligonucleotide probe confirmed the specificity of the amplifications.

irst
itation
f ıble 2

Results of cytokine receptor gene expression are presented in Table 2 (first citation of Table 2). Transcription of the gene encoding only a single subunit of the GM-CSFR or IL-3R was detected in a significant minority of cell lines (8/34, 23%). The alpha subunit of GM-CSFR was detected alone in 6/34 of the lines while the alpha subunit of IL-3R alone was not found in any. Both alpha subunits were identified in 2/34 cell lines. The beta subunit, common to both the GM-CSFR and IL-3R, was found as the sole transcript in 1/34 cell lines. Expression of a single

2023251797

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

subunit, or of only alpha subunits of both GM-CSFR and IL-3R, without concomitant production of beta subunits confers only low affinity binding to their respective cytokines. Simultaneous expression of both the alpha and beta subunits of either the GM-CSF or the IL-3 receptor was seen in 7/34 (20%) of the cell lines. Of these, transcripts of the two GM-CSFR subunits alone were detected in 1 and those of IL-3 in 2, while in 4 cell lines, both subunits of both receptors were identified. These included 2 small cell carcinoma cell lines, 1 melanoma cell line, 2 head and neck cancer cell lines, 1 ovarian carcinoma, and 1 renal carcinoma cell line. Transcription of the gene encoding the IL-6R was found in 13/34 (38%) cell lines including 2 small cell lung cancers, 2 melanomas, 4 head and neck tumors, 1 prostatic carcinoma, 1 gastric carcinoma, and 3 renal carcinomas. The expression of the IL-6 signal transducer, gp130, was expressed by all cell lines regardless of the concomitant transcription of the other cytokine receptors, including IL-6R.

ir t
itation
f ig.1

Characteristic results of PCR amplification of the panel of tumor cells are shown in Figure 1 (first citation of Fig.1). As indicated, a relatively high frequency of transcription of cytokine receptor genes was found. Amplification of even minute amounts of reverse-transcribed cDNA could conceivably yield false positive results. For this reason, we diluted the reverse-transcribed cDNA serving as template by 1:100 in those cell lines yielding initial positive PCR products. The diluted material was then amplified and the product quantitatively compared to that obtained from the PCR product using as template undiluted cDNA from the positive control cell lines by hybridizing to the corresponding internal oligonucleotide probe (not shown). Simultaneously run appropriate negative controls assured that positive samples did not result from contamination.

To test the in vitro proliferative effect of exogenously added growth factors on malignant cells, those cell lines expressing both subunits of either GM-CSFR, IL-3R, or those transcribing the single unit of IL-6R were cultured with their appropriate cytokine in a concentration range of 20 ng/ml to 500 ng/ml and stimulation was measured by incorporation of [^{3}H]-thymidine. No stimulatory effect as compared to the control culture was seen at any of the concentrations for either GM-CSF, IL-3 or IL-6 at either 3 or 6 days of culture (Table 3) (first citation of Table 3). The lower concentration of GM-CSF and IL-3 actually yielded diminished incorporation [^{3}H]-thymidine in cell line STO and DOZ suggesting that antiproliferative effect at those concentration

ir t
itation
f Table 3

2023251798

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

cannot be definitively ruled out.

By ELISA, neither GM-CSF nor IL-3 were detected in the supernatants of the cell line used in the proliferative assays. IL-6, as measured by bioassay with the IL-6-dependent murine hybridoma 7TD1 (33) is absent in the supernantants of lines ST0, D0Z and Detroit, while present in lines ADE and Hep-2 at 200 ng/ml and 225 ng/ml respectively.

Analysis by PCR amplification of the expression of the cytokine gene transcription corroborates these results. Transcripts of neither GM-CSF and IL-3 were not found in any cell line studied. However, transcription of the gene encoding IL-6 was detected in most cell lines from a variety of histologic origins (Table 4) (first citation of Table 4).

Fi st citation of Table 4

DISCUSSION

Hematopoietic growth factors have won an established place in the prevention and treatment of the hematologic complications induced by cancer chemotherapy. However, a number of reports have suggested that growth factors might also induce the in vitro proliferation of non-hematologic malignant cell lines or at least a subset of tumor cell lines (10-21). Presumably, the inappropriate presence of cell surface receptors for cytokines on tumor cells contibutes to the growth deregulation characteristic of malignancies. The use of growth factors, then, might facilitate the progression of the residual disease or, depending of the factor involved, might conceivably induce tumor cell differentiation as well.

In the present study, on examining the transcription of the genes encoding receptors for growth factors, we observed a relatively frequent expression of both subunits of either the GM-CSFR or the IL-3R (20%) as well as of the single subunit of IL-6R (38%). These included cell lines derived from a variety of malignant histologic types (Table I). Expression of the IL-6 signal transducer, gp130, was detected in all cell lines, even in those not transcribing the IL-6 receptor gene itself. This is consistent with previous observations on the ubiquity of gp130 expression (8). The ability of non-hematopoietic tumor cells to transcribe mRNA for receptors of normal hematopoietic growth factors suggests that the genes encoding those cell surface peptides are among the dysregulated genes that may be a marker of or even possibly causally linked to

2023251799

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

malignant proliferation. Significantly, the fact that some malignant cells dysregulate the gene encoding a single receptor subunit, while others dysregulate two genes points to the heterogeneity of tumors, even among those of the same histologic type (Table 2) regardless whether the receptor is functional or not. Further biological heterogeneity may be introduced by differential abilities to translate peptide products and assemble them on the cell surface as structurally and functionally the same receptor as present on normal hematopoietic cells. This forms the basis of further investigations (T. Guillaume et al, manuscript in preparation).

The sensitivity of PCR amplification could yield false positive results and thereby overestimate transcription of cytokine receptor genes. Generation of PCR product is geometrically proportional to the amount of template available for amplification (39). With the quantitative technique used in this study, even 1:100 dilutions of the reverse-transcribed cDNAs serving as PCR template yielded products detectable at the same level as detected with undiluted cDNA derived from the hematopoietic cell line serving as positive control. Had there been a systematic laboratory contaminant of samples actually negative for the receptor, one might have expected the dilutions to give product yields of varying intensity in quantitative PCR, depending on their degree of contamination. Furthermore, simultaneously run side-by-side negative controls were consistently negative. In fact, a majority of the simultaneously amplified malignant cell lines studied were negative for transcription of complete two-sub-unit cytokine receptors in the very sensitive oligonucleotide probe hybridizations used here, further arguing against indiscriminant, artifactual amplification. Basal levels of gene transcription of tissue-specific genes in non-specific cells, a phenomena termed illegitimate transcription, has been demonstrated by PCR amplification in a number of genes including β globin, Factor VIIIc, aldolase A, and anti-Müllerian hormone (40). It has not yet been demonstrated if it is a universal phenomena for all human genes. Even if true for the genes encoding cytokine receptors, it is reasonable to assume that as in the case of other tissue-specific gene, only those transcribed at a significant level may potentially produce biologically-active products. In the case of cytokine receptors, significant levels are those comparable to those in established cell line known to be cytokine-responsive which we have used as positive controls.

2023251800

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

Cell lines transcribing the complete receptors for either GM-CSF, IL-3 or IL-6 were examined in medium containing low concentration of FCS (3%) in the presence of a wide range of concentrations of cytokines, from 20 to 500 ng/ml, known to stimulate hematopoietic progenitor cell proliferation in vitro. No proliferative enhancement as measured by incorporation of [^{3}H]-thymidine could be detected at either 3 or 6 days of culture. In addition, at the lower concentrations of GM-CSF and IL-3 (20 and 100 ng/ml) decreased in [^{3}H]-thymidine incorporation was seen in cell lines STO and DOZ, so that an antiproliferative effect, at least on those two lines, cannot be entirely ruled out. These findings suggest that the growth factors neither induce previously non-clonogenic cells to clonal growth nor increase the size of existing clones. Since the cells themselves produce neither GM-CSF or IL-3, as determined both by the absence of gene transcription (Table 4) as well as by ELISA, the lack of response to exogenous GM-CSF or IL-3 is not due to occupation of all available cell surface receptor sites by endogenously produced factors. However, lines ST0, DOZ, ADE, Hep-2 and Detroit do transcribe the IL-6 gene (Table 4). Despite this, IL-6 activity, as measured by bioassay is absent in 3 (ST0, DOZ and Detroit) of these 5 lines. Since the bioassay requires intact cytokine, IL-6 gene transcription divorced from elaboration of the functional peptide product suggests either dysregulated translation, decreased cytoplasmic half-life, or a non-functional cytokine. As in IL-3 and GM-CSF, the absence of IL-6 production militates against the presence of an autocrine loop as an explanation for the lack of proliferative effect of exogenously added IL-6 in those lines. However, cell lines ADE and Hep-2 do produce detectable levels of IL-6 and their IL-6 receptor sites may already be occupied by endogenous cytokine. Conceivably, even small amounts of IL-6 could occupy sufficient surface receptor sites to block added IL-6 even in cell lines evidencing IL-6 mRNA transcription but no IL-6 production. However, in such a case, a disparity would remain between the strongly positive results on quantitative PCR determinations of mRNA and even a putatively low level of IL-6 production, below even detection by bioassay. Furthermore, the apparent discrepancy between cellular transcription of message encoding the cytokine receptors (Table 2) and the absence of proliferation in vitro on addition of cytokines (Table 3) suggests ineffective receptor peptide assembly despite production of full-length RNA message.

2023251801

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

Alternatively, low density of cytokine receptor peptides on the tumor cell surface may preclude effective binding of cytokine, or, even in the presence of adequate cytokine binding, the cell may lack the appropriate machinery for signal transduction.

Previous studies of the in vitro proliferative effects of GM-CSF on tumor cells have not been conclusive. While stimulations of fresh solid tumor biopsies including from breast, colon, lung, ovarian carcinomas as well as melanomas have been almost uniformly negative (13,14) proliferative responses to GM-CSF have been reported in established tumor cell lines, although variable results have been recorded between different laboratories, at times using the very same cell lines. That tumors of the same histopathology but of different sources might give variable results is not altogether surprising; malignancies, even of the same cell type, are by their nature biologically heterogeneous. In addition, heterogeneity of continually passed cell lines, even from the very same original source, can probably account for a great deal of apparently inconsistent in vitro results. The advantage of the present report is that cell lines used both in the cytokine receptor gene transcription study and in the proliferation assays include lines developed by us which have not undergone continual passage, and therefore are more likely to reflect in their biological activity the original tumors from which they were derived.

Studies regarding the in vitro effects of IL-3 on malignant growth have been less extensive than those for GM-CSF. Evidence exists, in a limited study, that IL-3 enhances the growth of pancreatic and gastric carcinoma cell lines and was additive to the stimulatory effect of GM-CSF (17). Likewise, IL-3 has been found to be stimulatory to a single colon carcinoma cell (11), and small cell lung carcinoma cell lines (18). In contrast to GM-CSF, IL-3 has also be found to be stimulatory in a minority (23%) of primary tumor biopsies in a tumor cloning assay (15).

Perhaps the most consistent observations have been made with IL-6. By immunochemical staining, IL-6 has been found to be present in a wide variety of solid tumors including colon, ovarian, and breast carcinomas as well as in Kaposi's sarcoma (41), in squamous cell carcinoma and in soft tissue sarcomas (42). However, such staining is unable to differentiate between endogenously produced tumor IL-6 and mere adhesion of cytokine produced elsewhere. On the other hand, renal carcinoma cell lines have been shown to both produce and proliferate in

2023251802

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

response to IL-6 and demonstrate growth arrest in the presence of anti-IL6 antibodies (43, 44). Expression of both IL-6R and IL-6 has been demonstrated in prostatic carcinoma (21), and while ovarian carcinoma cell lines have been seen to have both constitutional and inducible production of IL-6 (22, 42), addition of exogeneous IL-6 did not affect cell proliferation (45). It is unclear whether this means IL-6 has no growth effect on the tumor cells or whether endogenously produced IL-6 already occupied all available receptor sites. Similarly, elevated levels of IL-6 have been detected in human hepatic carcinoma cell lines (46).

In the present study, the presence of either an exterior cytokine paracrine effect or of self-sustaining autocrine loop in those malignant cells transcribing cytokine receptors is unlikely, given both the lack of cytokine production by the malignant cell themselves as measured by either ELISA or bioassay and the absence of proliferative response to exogeneously administered cytokines in vitro as measured by incorporation of [^{3}H]-thymidine. By design, the proliferative studies were carried out in flat-bottomed wells to avoid cellular confluence that might lead to contact inhibition and absence of confluence was ascertained visually. Proliferative studies depend on the cells being in active growth phase. The concentration of cells (10^5/ml or 10^4/100 μl well), the range of added cytokines (20-500 ng/ml) and the incubation period (3 and 6 days) are in keeping with previous cellular proliferative studies (10,16,17,18,19). In most other studies, as in the present one, growth factors were added only at the beginning of the culture period; only in the study of Dippold et al. (17) were growth factors added initially and every other day. Here, as in all published reports, conclusions regarding cellular proliferation are limited to the conditions used Even if present, an in vitro stimulatory effect of cytokines on malignant cells would not necessarily translate into enhanced cell growth in vivo. Berdel et al (47) have demonstrated that two malignant cell lines, respond to exogeneous GM-CSF and IL-3 by enhanced growth in vitro. However, when xenotransplanted in nude mice, the two malignant cells lines were not induced by those same growth factors administered in vivo to the mice hosts. In vitro growth stimulation may in general not be easily extrapolatable to in vivo tumors. Apparent inconsistencies between the two might arise from differing pharmacokinetics of cytokines in vitro and in vivo. In addition, there is inherent heterogeneity of in vivo tumors even as compared to in vitro tumor cell lines,

2023251803

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

and the same clonogenic subpopulation present in vitro may not be the one predominating in vivo. Furthermore, apart from any direct effect of cytokine on malignant cells, growth factors can independently promote antitumor toxicity in vivo. The systemic administration of IL-6 in vivo has been shown to mediate reductions in pulmonary and hepatic metastases in syngeneic murine tumors (48,49) which appears to be due to in vivo generation of tumor-specific cytotoxic T cells at the tumor site. In fact, unlike the other cytokines studied here, IL-6 has been seen to directly inhibit malignant cells : In vitro, IL-6 has been shown to suppress the proliferation of human breast carcinoma and lymphoma cell lines (50).

In vitro experimental evidence gathered to date has suggested the possibility of in vitro stimulation of at least a subgroup of malignant cell types by cytokines. Our own findings confirm the ability of a significant minority of tumor cells to transcribe the genes encoding cell surface cytokine receptors. However, the lack of in vitro cellular proliferation to exogenously provided growth factors, together with the absence of endogenous production of cytokines suggests that even though possessing the ability to transcribe message for cytokine receptors, the malignant cell line studied here, derived from a variety histologic cell types, lack the cellular machinery necessary to respond to growth factors. This in turn suggests that in vivo therapeutic administration of cytokines in patients with solid tumors may entail no any additional risk of stimulating tumor regrowth.

2023251804

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

ACKNOWLEDGMENTS

We wish to thank Dr. J. De Grève for providing the ovarian cancer cell lines, Dr. F. Houssiau for performing the IL-6 bioassays and Mrs. Gilda Manicourt for her editorial assistance in preparing the manuscript.

2023251805

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

REFERENCES

1. Oster, W., and Schulz, G. Interleukin 3 : biological and clinical effects. Int. J. Cell Cloning, 9: 5-23, 1991.

2. Gasson, J.C. Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood, 77: 1131-1145, 1991.

3. Gearing, D.P., King, J.A., Gough, N.M., and Nicola, N.A. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. The Embo J., 8: 3667-3676, 1989.

4. Kitamura, T., Sato, N., Arai, K.I., and Miyajima, A. Expression cloning of the human IL-3 receptor cDNA reveals a shared β subunit for the human IL-3 and GM-CSF receptors. Cell, 66: 1165-1174, 1991.

5. Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K.I., Yokota, T. and Miyajima, A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF) : Reconstitution of a high-affinity GM-CSF receptor. Proc.Natl.Acad. Sci. USA, 87: 9655-9659, 1990.

6. Van Snick, J. Interleukin-6 : an overview. Annu. Rev. Immunol., 8: 253-278, 1990.

7. Yamasaki, K. Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., Kishimoto, T. Cloning and expression of the human interleukin-6 (BSF/IFNβ2) receptor. Science, 24: 825-828, 1988.

8. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell, 58: 573-581, 1989.

9. Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. Molecular cloning and expression of an IL-6 signal transducer, gp 130. Cell, 63: 1149-1157, 1990.

10. Dedhar, S., Gaboury, L., Galloway, P., and Eaves, C. Human granulocyte-macrophage colony-stimulating factor in a growth factor active on a variety of cell types of non-

2023251806

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

hematopoietic origin. Proc. Natl. Acad. Sci USA, 85: 9253-9257, 1988.

11. Berdel, W.E., Denheuser-Riedl, S., Steinhauser, G., and Winton, EF. Various human hematopoietic growth factor (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of non-hematopoietic tumor cells. Blood, 73: 80-83, 1989.

12. Baldwin, G.C., Gasson, J.C., Kaufman, S.E., Quen, S.G., Williams, R.E., Avalos, B.R., Gazdar, A.F., Golde, D.W., and Dipersio, J.F. Non hematopoietic tumor cells express functional GM-CSF receptors. Blood, 73: 1033-1037, 1989.

13. Salmon, S.E., and Liu, R. Effects of granulocyte-macrophage colony-stimulating factor on in vitro growth of human solid tumors. J Clin Oncol, 7:1346-1350, 1989.

14. Foulke, R.S., Marshall, M.H., Trotta, P.P., and Von Hoff, D.D. In vitro assessment of the effects of granulocyte-macrophage colony-stimulating factor on primary human tumors and derived lines. Cancer Res, 50: 6264-6267, 1990.

15. Joraschkewitz, M., Depenbrock, H., Freund, M., Erdmann, G., Meyer, J.H., De Riese, W., Neukam, D., Hanauske, U., Krumwieh, M., Polidowa, H., Hanauske, A.R. Effects of cytokines on in vitro colony formation of primary human tumor specimens. Eur J Cancer, 26: 1070-1074, 1990.

16. Serve, H., Steinhauser, G., Oberberg, D., Flegel, W.A., Northoff, H., Berdel, W.E. Studies on the interaction between interleukin 6 and human malignant non-hematopoietic cell lines. Cancer Res., 51:3862-3866, 1991.

17. Dippold, W.G., Klingel, R., Kerlin, M., Schwaeble, W., and Meyer Zum Büschenfelde, K.H. Stimulation of pancreas and gastric carcinoma cell growth by interleukin 3 and granulocyte-macrophage colony-stimulating factor. Gastroenterology, 100: 1338-1344, 1991.

18. Vellenga, E., Biesma, B., Meyer, C., Wegteveld, L., Esselink, M., and de Vries, E.G.E. The effects of five hematopoietic growth factors on human small cell lung carcinoma cell lines : interleukin 3 enhances the proliferation in one of the eleven cell lines. Cancer Res, 51: 73-76, 1991.

2023251807

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

19. Twentyman, P.R., and Wright, K.A. Failure of GM-CSF to influence the growth of small cell and non-small cell lung cancer cell lines in vitro. Eur J Cancer, 27: 6-8, 1991.

20. Members of the EORTC clonogenic assay screening study group. Human granulocyte-macrophage colony-stimulating factor modulates in vitro growth in only a minority of continuous human tumor cell lines. Eur. J. Cancer, 27: 231-235, 1991.

21. Siegall, C.B., Schwab, G., Nordan, R.P., Fitzgerald, D.J., and Pastan, I. Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. Cancer Res, 50: 7786-7788, 1990.

22. Malik, S., and Balkwill, F. Epithelial ovarian cancer : a cytokine propelled disease ? Br. J. Cancer, 64: 617-620, 1991.

23. Martinez-Maza, O., and Berck, J.S. Interleukin-6 and cancer treatment. In vivo, 5:583-588, 1991.

24. Hamblin, A.S. Lymphokines. pp. 1-12. Oxford : IRL Press, 1988

25. Symann, M. Hematopoietic growth factors as supportive therapy for cancer- and chemotherapy-induced conditions. Curr. Opinion Oncol., 3: 648-655, 1991.

26. Simms, E., Gazdar, A.F., Abrams, P.G., and Minna, J.D. Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor-supplemented medium. Cancer Res., 40: 4356-4363, 1980.

27. Gallagher, R., Collins, S., Trujillo, J., Mc Credie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Rucetti, F., and Gallo, R. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood, 54: 713-718, 1979.

28. Koeffler, H.P., and Golde, D.W. Acute myelogenous leukemia : a human cell line responsive to colony-stimulating activity. Science, 200: 1153-1154, 1978.

29. Muraguchi, A., Kishimoto, T., Miki, Y., Kuritani, T., Kaieda, T., Yoshizaki, K., and Yamamura, Y. T-cell replacing factor (TRF) induced IgG secretion in a human B blastoid cell line and demonstration of receptors for TRF. J Immunol, 127: 412-416, 1981.

2023251808

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

30. Kirby, K.S. Isolation of nucleic acids with phenolic solvents. Meth Enzymol, 12 B: 87-98, 1968.

31. Chirgwin, J.J., Przbyla, A.E., Mac Donald, R.J., and Rutter, W.J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18: 5294-5299, 1979.

32. Guillaume, T., Rubinstein, D.B., Young, F., Tucker, L., Logtenberg, T., Schwartz, R.S., and Barrett, K.J. Individual VH genes detected with oligonucleotide probes from the complementary-determining regions. J Immunol, 145: 1934-1945, 1990.

33. Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P.G., Rubira, M.R., and Sympson, R.J. Purification and NH2-terminal aminoacid sequence of a T-cell derived lymphokine with growth factor activity for B-cell hybridomas. Proc. Natl. Acad. Sci. USA, 83:9679-9683, 1986.

34. Lee, F., Yokota, T., Otsuka, T., Gemmel, L., Larson, N., Luh, J., Arai, K.I., Rennick, D. Isolation of cDNA for a human granulocyte-macrophage colony-stimulating factor by functional expression in mammalian cells. Proc. Natl. Acad. Sci. USA, 82:4360-4364, 1985.

35. Yang, Y.-C., Ciarletta, A.B., Temple, P.A., Chung, M.P., Kovacic, S., Witek-Giannotti, J.S., Leavy, A.C., Kriz, R., Donahue, R.E., Wong, G.G., Clark, S.C. Human IL-3 (multi-CSF) : identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell, 47:3-10, 1986.

36. Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsumasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., Kishimoto, T. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature, 324:73-76, 1986.

37. Ponte, P., Ng, S-Y., Gunning, P., and Kedes, L. Evolutionary conservation in the untranslated regions of actin mRNAs : DNA sequence of human beta-actin cDNA. Nucl. Acids Res., 12: 1687-1696, 1984.

38. Hibi, K., Takahashi, T., Sekido, Y., Ueda, R., Hida, T., Ariyoshi, Y., Takagi, H., and

2023251809

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

Takahashi, T. Co-expression of the stem cell factor and the c-kit genes in small cell lung cancer. Oncogene, 6: 2291-2296, 1991.

39. Saiki, R.K., Gelfand, D.H., Stoffel, S., Sehorf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science, 239: 487-491, 1988.

40. Chelly, J., Concordet, J.P., Kaplan, J.C., and Kahn, A. Illegitimate transcription : transcription of any gene in any cell type. Proc. Natl. Acad. Sci. USA, 86: 2617-2621, 1989.

41. Tabibzadeh, S.S., Poubouridis, D., May, L.T., and Sehgal, P.B. Interleukin-6 immunoreactivity in human tumors. Am J Pathol, 135: 427-433, 1989.

42. Miki, S., Iwano, M., Miki, Y., Yamamoto, M., Tang, B., Yokokawa, K., Sonoda, T., Hirano, T., and Kishimoto, T. Interleukin-6 functions as an in vitro autocrine growth factor in renal cell carcinomas. FEBS Lett., 250: 607-610, 1989.

43. Gruss, H.J., Brach, M.A., Mertelsmann, R.H., and Hermann, F. Interferon-gamma interrupts autocrine growth mediated by endogenous interleukin-6 in renal-cell carcinoma. Int. J. Cancer, 49: 770-773, 1991.

44. Breen, E.C., Rezai, A.R., Nakajima, K., Beall, G.N., Mitsuaysu, R.T., Hirano, T., Kishimoto, T., and Martinez-Maza, O. Infection with HIV is associated with elevated IL-6 levels and production. J Immunol, 144 : 480-484, 1990.

45. Watson, J.M., Sensintaffar, J.L., Berek, J.S., and Martinez-Maza, O. Epithelial ovarian cancer cells constitutively produced interleukin-6. Cancer Res, 50: 6959-6965, 1990.

46. Matsuguchi, T., Okamura, S., Kourasaki, C., and Niho, Y. Production of interleukin-6 from human liver cell lines : production of interleukin 6 is not concurrent with the production of alpha-fetoprotein. Cancer Res., 50: 7457-7459, 1990.

47. Berdel, W.E., Zafferassi, M., Serrekowitsch, R., Kreuser, E.D., and Thich, E. Effect of interleukin-3 and granulocyte-macrophage colony-stimulating factor on growth of xenotransplanted human tumour cell lines in nude mice. Eur J Cancer, 28: 377-380, 1992.

2023251810

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

48. Mulé, J.J., McIntosh, J.K., Jablons, D.M., and Rosenberg, S.A. Antitumor activity of recombinant interleukin 6 in mice. J. Exp. Med., 171:629-636, 1990.

49. Mulé, J.J., Custer, M.C., Travis, W.D., and Rosenberg, S.A. Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J. Immunol., 148:2622-2629, 1992.

50. Chen, L., Mori, Y., Zilberstein, A., and Revel, M. Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interferon-β2. Proc. Natl. Acad. Sci. USA, 85:8037-8041, 1988.

2023251811

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

Fig. 1. PCR amplification of genes encoding cytokine receptors. cDNA was prepared by reverse transcription of total cellular RNA derived from malignant cell lines. The product was used as template for 25 cycles of PCR amplification with cytokine receptor gene-specific primers (Table 1). PCR products were size fractionated on 2% agarose gels, blotted onto nylon membranes and hybridized to internal oligonucleotide probes end-labelled with T4 polynucleotide kinase and gamma ^{32}[P]-ATP. Molecular weights are indicated to the right.

2023251812

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

TABLE 1 Primers used for PCR amplification and internal oligonucleotide probes for detection of PCR products

PCR PRIMERS			LENGTH OF AMPLIFICATION PRODUCT	REFERENCE
GM-CSFR α	sense	5'-CTTCTCTCTGACCAGCA-3'	530 bp	[3]
	anti-sense	5'-ACATGGGTTCCTGAGTC-3'		
GM-CSFR β	sense	5'-TCACTCCACTCGCTCCAGAT-3'	316 bp	[5]
	anti-sense	5'-AATACATCGTCTCTGTTCAG-3'		
IL-3R α	sense	5'-ATGTGACCGATATCGAGTGTG-3'	442 bp	[4]
	anti-sense	5'-GATACCGAAGGCTGCGCTCCT-3'		
IL-6R	sense	5'-TCCACGACTCTGGAAACTAT-3'	306 bp	[7]
	anti-sense	5'-ACTATGTAGAAAGAGCTGTC-3		
gp130	sense	5'-ATTCCTAAGGAGCAATATAC-3'	759 bp	[9]
	anti-sense	5'-TGGTCTATCTTCATAGGTGT-3'		
GM-CSF	sense	5'-TGGCCTGCAGCATCTCTGCA-3'	322 bp	[34]
	anti-sense	5'-GTGATAATCTGGGTTGCACA-3'		
IL-3	sense	5'-AGACAAGGTCCTTGAAGACA-3'	344 bp	[35]
	anti-sense	5'-CTCAAGGGTTTTCAGATAGA-3'		
IL-6	sense	5'-CAGGAGAAGATTCCAAAGAT-3'	459 bp	[36]
	anti-sense	5'-ACTGGTTCTGTGCCTGCAGC-3'		
βactin	sense	5'-GTGGGGCGCCCAGGCACCA-3'	519 bp	[37]
	anti-sense	5'-TCCTTAATGTCAAGAACGAT-3'		

INTERNAL OLIGONUCLEOTIDE PROBES		T° HYBRIDIZATION	REFERENCE
GM-CSFR α	5'-TGTGACTCCTTCATGCAGAC-3'	55°C	[3]
GM-CSFR β	5'-GTGTGGTCTATGTGTTCGTA-3'	55°C	[5]
IL-3R α	5'-AAGAAATCCACGTCATGAAT-3'	50°C	[4]
IL-6R	5'-ATCCACAAACAACATTGCTG-3'	50°C	[7]
gp130	5'-GAATAATCAACAGTGCATGA-3'	50°C	[9]
GM-CSF	5'-ATTTCTGAGATGACTTCTAC-3'	55°C	[34]
IL-3	5'-CTCAAGGGTTTTCAGATAGA-3'	55°C	[35]
IL-6	5'-GTCTCCTCATTGAATCCAGA-3'	55°C	[36]
βactin	5'-AGGTCTCAAACATGATCTGG-3'	50°C	[37]

20232518 13

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

TABLE 2 *Detection of cytokine receptor gene transcription by PCR amplification of reverse-transcribed total RNA derived from human malignant cell lines.*

	GM-CSFRα	GM-CSFRβ	IL-3Rα	IL-6R	gp130
HL-60	+	+	-	-	+
KGI	+	+	+	-	+
CESS	+	+	+	+	+
Small cell lung cancer					
H69	-	-	-	-	+
H128	-	-	-	-	+
OC1	-	-	-	-	+
OC2	-	-	-	-	+
OC3	-	-	-	-	+
FRA	-	-	-	-	+
STO	-	+	+	+	+
GIL	-	+	-	-	+
THO	+	-	-	-	+
BAT	-	-	-	-	+
DEV	-	-	-	-	+
VHE	+	-	-	-	+
FIC	-	-	-	-	+
DOZ	-	+	+	+	+
Melanoma					
DES	+	-	-	+	+
G43	-	-	-	-	+
WO	-	-	-	-	+
RENI	-	-	-	-	+
ADE	+	+	+	+	+
Head and neck cancer					
Fadu	+	-	+	+	+
KB	+	-	-	+	+
Hep-2	+	+	-	+	+
Detroit	+	+	+	+	+
RPMI 2650	-	-	-	-	+
Ovarian cancer					
AZ 224	+	-	-	-	+
AZ 382	+	+	+	-	+
AZ 364	-	-	-	-	+
AZ 303	+	-	+	-	+
Prostatic cancer					
DU145	+	-	+	+	+
Gastric cancer					
AGS	-	-	-	-	+
KATO III	+	-	-	+	+
Kidney carcinoma					
CAKI I	+	+	+	+	+
CAKI II	+	-	-	+	+
A498	-	-	-	+	+

2023251814

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

TABLE 3 *[^{3}H]-thymidine incorporation by selected malignant cell lines. 10^5 cells/ml were cultured in 0, 20, 100, 500 ng/ml of either human recombinant GM-CSF, IL-3 or IL-6. Incorporations shown represent mean cpm ± SEM of triplicate determinations following 6 days of culture. Concentrations of added growth factors are indicated to the right.*

CELL LINE	GM-CSF	IL-3	IL-6	
STO	1,855±453	1,219±19	1,515±152	0 ng/ml
	1,238±83	957±27	1,319±87	20
	1,689±242	1,656±103	1,788±194	100
	1,498±104	1,243±101	1,793±182	500
DOZ	95,929±1,181	97,556±2,693	113,510±764	0 ng/ml
	25,828±3,448	43,349±2,297	126,253±2,681	20
	69,057±1,174	95,911±2,405	118,358±3,228	100
	106,901±4,085	99,249±2,512	146,272±2,000	500
DES	75,070±2,044	87,981±13,540	100,406±7,922	0 ng/ml
	106,421±3,056	117,698±2,338	138,621±2,692	20
	113,433±4,797	110,762±1,483	107,408±2,203	100
	90,155±3,565	120,166±5,908	63,294±8,425	500
ADE	5,482±466	7,397±894	6,576±727	0 ng/ml
	8,978±1,374	9,162±104	9,652±1,419	20
	8,482±705	9,109±104	6,385±93	100
	6,624±407	7,494±176	9,133±555	500
Hep-2	16,774±431	17,888±255	18,089±3,225	0 ng/ml
	12,659±682	16,275±463	26,812±311	20
	13,141±108	18,457±581	29,779±352	100
	14,400±1,309	19,963±1,646	24,643±1,186	500
Detroit	30,368±1,221	42,428±8,135	45,904±4,589	0 ng/ml
	35,720±1,225	47,487±5,722	51,268±1,538	20
	39,203±6,238	57,645±6,118	56,484±7,263	100
	33,876±5,782	48,221±2,274	45,719±2,657	500

2023251815

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

TABLE 4 *Detection of cytokine gene transcription by PCR amplification of reverse-transcribed total RNA derived from human malignant cel lines.*

	GM-CSF	IL-3	IL-6
ST0	-	-	+
DOZ	-	-	+
DES	-	-	-
ADE	-	-	+
Fadu	-	-	+
Hep-2	-	-	+
Detroit 562	-	-	+
AZ382	-	-	+
DU145	-	-	+
KATOIII	-	-	-
CAKII	-	-	+
CAKIII	-	-	+
A498	-	-	+

2023251816

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000

20232518 17

Source: https://www.industrydocuments.ucsf.edu/docs/jflm0000